REVIEW ARTICLE

Chemical and physical basics of routine formaldehyde fixation

Rooban Thavarajah, Vidya Kazhiyur Mudimbaimannar, Joshua Elizabeth, Umadevi Krishnamohan Rao, Kannan Ranganathan

Departments of Oral and Maxillofacial Pathology, Ragas Dental College and Hospital, Uthandi, Chennai, India

Address for correspondence:
Dr. Rooban Thavarajah,
Department of Oral and Maxillofacial Pathology,
Ragas Dental College and Hospital,
Chennai, India.
E-mail: t.rooban@rediffmail.com

ABSTRACT

Formaldehyde is the widely employed fixative that has been studied for decades. The chemistry of fixation has been studied widely since the early 20th century. However, very few studies have been focused on the actual physics/chemistry aspect of process of this fixation. This article attempts to explain the chemistry of formaldehyde fixation and also to study the physical aspects involved in the fixation. The factors involved in the fixation process are discussed using well documented mathematical and physical formulae. The deeper understanding of these factors will enable pathologist to optimize the factors and use them in their favor.

Key words: Fixation, Antigen retrieval, buffered formalin, diffusion, formaldehyde, immunohistochemistry, penetration

INTRODUCTION

Fixation is a crucial step in processing of biopsy tissue specimen for the examination and archival preservation. Fixation helps to preserve cellular architecture and composition of cells in the tissue to allow them to withstand subsequent processing. Fixation also preserves the proteins, carbohydrate and other bio-active moieties in their spatial relationship to the cell, so that they can be studied.[1]

An ideal fixative is expected to harden (to impart mechanical rigidity to withstand tissue processing) tissue components and prevent decomposition, putrefaction, and autolysis. Fixation is a physico-chemical process that is gradual and complex, involving diffusion of fixative into the tissue and a variety of potential physical phenomenon and chemical reactions. To date, no ideal fixative has been found, i.e., a fixative that perfectly preserves cellular morphology and yet does not modify the specimen composition so as not to change the reactivity of the chemical moieties therein for subsequent detection. Because of this issue, the selection of a particular fixative generally warrants multiple and careful considerations.[1,2] Unless penetration occurs, fixation is not possible.

Access this article online

Quick Response Code:

Website: www.jomfp.in

DOI: 10.4103/0973-029X.102496

There are 4 major groups of fixatives, namely the aldehydes, oxidizing agents, alcohol based fixatives and the metallic group of fixatives. The aldehydes (formaldehyde, glutaraldehyde) and oxidizing agents (osmium tetraoxide, potassium permanganate) acts by cross-linking proteins. Alcohol based fixatives (methyl alcohol, ethyl alcohol, acetic acid) are protein-denaturing agents. Metallic group of fixatives acts by forming insoluble metallic precipitates like mercuric chloride and picric acid. The choice of the fixative is based on tissue and anticipated ancillary tests.[3]

Formalin is the widely used fixative in pathology labs worldwide owing to its convenience in handling, high degree of accuracy and extreme adaptability. The basics of chemical reactions involved in formalin fixation have been described in literature.[4,5] Penetration of formalin in to the specimen is a physical process by which the solution diffuses in to the specimen to reach the innermost layers of cells. This movement of formalin is governed by several physical factors.[3] The aim of this review is to attempt to revisit the chemical basis of formalin fixation, as well as to explain the effects of various factors on formalin fixation using known physical equations and basic chemical reactions.

CHEMICAL BASICS OF FORMALDEHYDE FIXATION

Proteins are basic blocks of any tissue. Protein structures can be classified into 4 levels of structural organization. Primary level is the amino acid structure. The remaining secondary, tertiary and quaternary structure refers to the peptide arrangement in the polypeptide backbone, three

dimensional structure of globular protein and structural aggregates of globular proteins respectively. Formaldehyde reacts with primary amines to form Schiff bases, with amides to form hydroxymethyl compounds. Hydroxymethyl groups condense with another amide moiety to form methyl daimides. Alcoholic hydroxyl forms acetals while sulfhydro groups form sulfhydral acetal analogues with formaldehyde.[6] Based on the affinity to combine with formaldehyde, proteins could be classified as strong affinity, moderate and less affinity ones. Presences of tyrosine rings, in proteins, have been identified as an important factor for the affinity of the protein to formaldehyde. In its absence, the presence of arginine residue, phenylalanine or tryptophan as a conserved substitution (tyrosine to a phenylalanine or tryptophan) would help to create the formalin affinity.[4]

The routinely used fixative is 10% formalin, which is 3.7% formaldehyde in water with 1% methanol. It is an inexpensive, commonly available fixative that does not cause excessive tissue shrinkage or distortion of cellular structure. The undiluted commercial formaldehyde solutions contain 10% methanol as a preservative to prevent the spontaneous condensation reaction.[1] Owing to this, the commercial formalin is a 2-phase fixative, with an initial alcohol fixation phase, followed by a cross-linking phase mediated by aldehyde. The alcohol initially causes dehydration in the process, hardening the tissues and membrane. Formalin, when stored for longer periods, gets oxidized to form formic acid. Hence in stored formaldehyde, presence of unknown formic acid (also reacts with blood to form a birefringent crystal called formalin pigments) is expected.[1]

Formaldehyde, in aqueous solution, becomes hydrated to form a glycol (hydrated formaldehyde) called methylene glycol. Methylene glycol hydrate molecules react with one another, combining to form polymers [Figures 1 and 2].[5] They can be given by the equation:

$CH_2O + H_2O \leftrightarrows CH_2(OH)_2$ (Methylene Glycol).

$nCH_2(OH)_2 \leftrightarrows H_2O + HO(CH_2O)_nH$ (Polyoxy methylene Glycol).

Long standing Methylene glycol polymerizes to form polyoxymethylene glycol. In a neutral to alkaline buffered system such as tissues, it depolymerizes to methylene glycol which dehydrates into carbonyl formaldehyde (one that contain C with a double bond with O, dehydrated form). Both the hydrated and non-hydrated forms of formaldehyde fix the tissue.[1,3,5]

In an ideal stable solution, the equilibrium (the state in which the right and left half of the equation are symmetrical and balanced) between methylene glycol and formaldehyde in aqueous solution lies in favor of methylene glycol. The conversion of methylene glycol to active carbonyl formaldehyde can be accomplished only by removal of formaldehyde. Infrared spectra of commercial formaldehyde solutions show no evidence of carbonyl formaldehyde.[1,6] Similarly, the major spikes or lines of the Raman spectrum correspond to those of methylene glycol only.[7]

When tissues are immersed in formalin, they are rapidly penetrated by methylene glycol and the minor quantity formaldehyde. Actual covalent chemical reaction of the fixative solution with tissue depends on the formaldehyde present being consumed after forming bonds with the tissue components. Consecutively, more formaldehyde forming from dissociation of methylene glycol leading to shift of the equation, so more formaldehyde is formed.[1,5,6]

Formaldehyde, being a reactive electrophilic species, it reacts readily with various functional groups of biological macromolecules in a cross-linking fashion [Figure 3]. Studies indicate that the most frequent type of cross-link formed by formaldehyde in collagen is between the nitrogen atom at the end of the side-chain of lysine and the nitrogen atom of a peptide linkage and the number of such cross-links increases

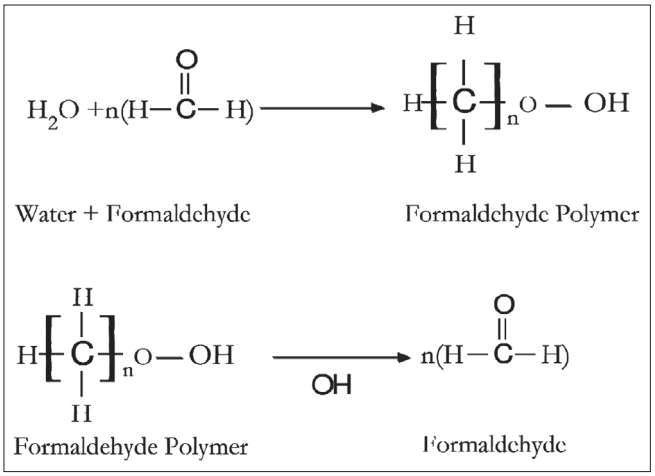

Figure 1: Reaction of formaldehyde

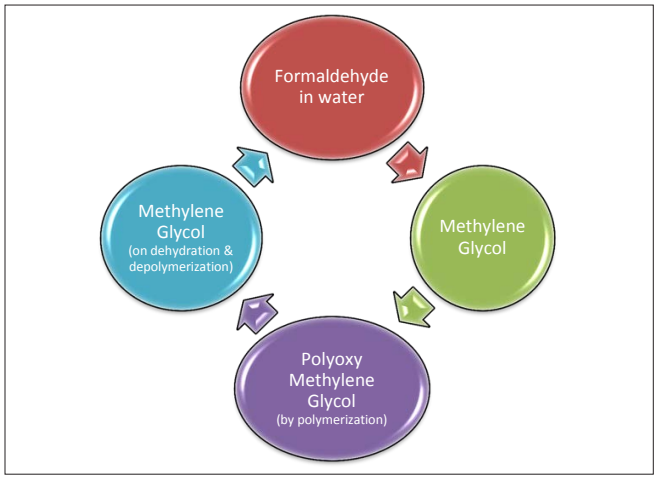

Figure 2: Cycle of aqueous formaldehyde

with time.[1,6]

In the initial or primary stage, reactive sites are primary amines (lysine), purines and thiols (cysteine) forming mono, dimethylol or hydroxyl methyl derivatives that are covalently bound to tissue [Figure 3.1]. The subsequent cross-linking occurs by formation of a cross-link of -CH2- called a methylene bridge. [Figure 3.2] They proceed to involve the other functional groups such as amides, asparagines and guanidine and tyrosine carbon rings.[1,3,5,6] Formaldehyde reacts with an amine creating a reactive iminium ion intermediate. The iminium ion then reacts with the phenol group of tyrosine, creating a covalent bond.[4]

The initial cross linking is completed by 24 h to 48 h after penetration while the latter may take about 30 days to generate the stable covalent cross linkages. The initial phase of the reaction is reversible while at the latter stages the reaction becomes irreversible when there is high number of covalent bonds formed. This alters the physicochemical state of tissue such as redox and membrane potentials of the tissue, surface charges and thereby it changes the reactivity of cellular components. However the minimum length of time required for both such complex interactions to become completely over is yet to be determined. The reversible nature of the initial phase reaction is the basis of antigen retrieval in molecular techniques.[4-6]

When formaldehyde reaches a cell by diffusion as methylene glycol and breaks down (by shift in equation), they experience a period when there is an increasing amount of diffused formaldehyde in the cell. Initially the remnant aldehyde dehydrogenases in the cell act on formaldehyde and metabolize it. After this, there is a rapid influx of formaldehyde into tissue as formaldehyde starts to react with proteins. Already fixed tissue may act as a barrier to this influx.[8]

Biopsy specimen consists of a system of biological structures and membranes with varying degrees of susceptibility to osmotic forces. Osmotic properties of a solution may be

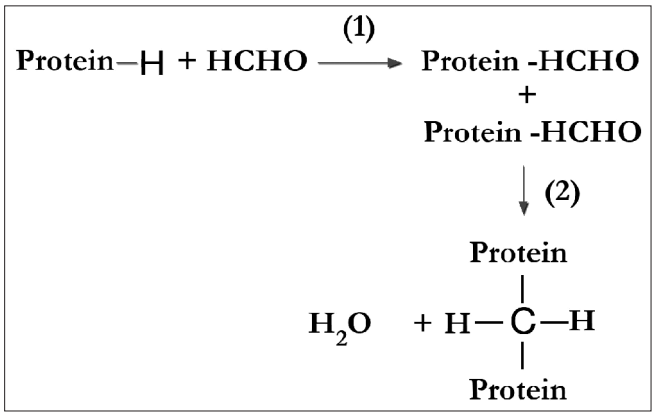

Figure 3: Protein Interaction of Formaldehyde (1) Initial reaction (2) Late reaction

expressed as the moles of molecules or ions dissolved in a liter of solvent. A "10% formalin" or 4% formaldehyde solution is 1.3 molar by definition. The completely unbuffered solution of formaldehyde, without methanol preservative, exerts an osmotic pressure in range of 1300 mO under standard conditions. By comparison, isotonic salt solutions have osmolarities on the order of 250-350 mO. Hence, formalin is expected to diffuse into tissue faster. Also, as a small molecule, formaldehyde (molecular weight 30) is expected to penetrate tissues at a rate independent of concentration and fix the tissue very fast. However in reality, the fixation takes a longer time. This discrepancy is referred to in literature as penetration-fixation paradox. Formaldehyde solution penetrates faster but takes longer time to fix. The answer to this lies in the chemistry of formaldehyde fixation and the rate to shift the equilibrium of formaldehyde liberation from polymers of methylene glycol.[1] Hence, it is recommended to use a freshly prepared solution of formalin to keep the concentrations of polymers low.

Factors influencing formaldehyde fixation

As discussed, there are two components in formaldehyde fixation-penetration and fixation. Penetration refers to the ability of the solution to diffuse into the tissue while fixation is the ability of the formaldehyde to complete the initial cross linking. For routine histochemical process, it is advised that the tissue blocks thickness be in the range of 20 mm so that the penetration occurs by 24 h at 25°C or 18 h at 37°C.[1]

Variation in factors such as buffering capacity, depth of penetration, temperature, concentration and time interval would influence the completion of fixation. In tissue level, there are more factors that would influence the penetration and fixation.

Factors influencing penetration

Literature has sufficient evidence to prove that penetration of formalin in tissue is governed by Fick's law of diffusion.[9] It has been repeatedly studied and reported about the various physical factors that influence the rate of penetration.

If formaldehyde is assumed to be an exact equilibrium where there is no shift in concentration of the gradients, then diffusion or penetration can be explained by Fick's first of law of diffusion.[10,11] The equation states that:

$$\text{Diffusion Flux } J_i = \frac{DC_i \partial_{\mu i}}{RT\ \partial x} \qquad \text{(Equation 1)}$$

Where i is species considered
D is diffusion coefficient or the constant
C is concentration in mol/m^3
R is universal gas constant in J/K mol
T is absolute temperature (in Kelvin)
μ_i is chemical potential of the species
x is length

In this above mentioned formula, D or the diffusion constant is directly proportional to square root of velocity and is influenced by temperature, viscosity of fluid and size of the particle and correlated by the Stokes-Einstein law for solutions with low Reynolds numbers[11,12] that is as given below:

$$\text{Diffusion Constant } D = \frac{K_B T}{6\pi\eta r} \quad \text{(Equation 2)}$$

(or)

$$\text{Diffusion Constant } D = \mu K_B T \quad \text{(Equation 3)}$$

Where K_B is Boltzmann's constant ($1.3806488 \times 10^{-23}$)

T is absolute temperature (in Kelvin)

η is viscosity and

r is radius

μ is particle's terminal drift velocity to an applied force

If one were to consider that the concentration of formaldehyde, at micro-levels, varies widely continuously, then Fick's second law of diffusion need to be applied.[10,11]

$$\frac{\partial\phi}{\partial t} = \Delta(D\nabla\phi) \quad \text{(Equation 4)}$$

Where φ = concentration in dimension, amount of substance / length^3 (mol/m^3)

t = time

Δ= gradient operator

D = diffusion coefficient

From these equations it can be safely concluded that penetration is dependent on the following:

1. Temperature has a dual role. It is inversely related to influx (equation 1) while directly being related to Diffusion constant (equation 2). The temperature used here is in Kelvin. A raise in 1°C would increase the Kelvin scale by 1 and elucidate a change of 0.37. (Conversion scale for Kelvin is temperature in °C + 273.15) Impact of temperature will directly correlate with Brownian movement of fluid which would affect the drift velocity. Radius of the active molecule will also be influenced by the increase in temperature.
   a. The density of aqueous formaldehyde as a function of temperature had been described by the formula[13]

$$\rho_m = \rho_w + (5.0950 - 6.8166 \times 10^{-3} T) W_F \quad \text{(Equation 5)}$$

   Where ρ_m is density of aqueous formaldehyde solution
   ρ_W is density of water
   T is absolute temperature in Kelvin
   W_F is overall weight% of formaldehyde in the solution.

   In this equation it is observed that density of the formaldehyde solution is linearly associated with the weight% of formaldehyde of the solution and also an association of temperature.
   b. The viscosity of aqueous formaldehyde as a function of temperature has been described in literature. The residual of equation did not reveal any clear trend of viscosity as a function of W_F or temperature.[13]
2. pH has a major role to play in the dissociation of the solution in equilibrium.[14] Alkaline pH and or presence of hydroxyl ions play an important role in conversion of polyoxymethylene glycol. In a mild alkaline system it depolymerizes to methylene glycol which dehydrates to maintain equilibrium with active carbonyl formaldehyde. Presence of formic acid in long standing solution will appear to retard this reaction. Fixant such as acid formalin will retard this depolymerization. However, this does not occur with freshly prepared solutions of formaldehyde.[1,7,14] The other implication is the advocacy of using buffered formalin. Buffered formalin offers resistance to minor changes in pH, hence negating the minor changes of pH.
   It is reported that at room temperature, unbuffered formalin has pH in range of 4, as many amino and guanidyl groups carry H^+ charge, formaldehyde can react only with limited available uncharged groups. With time, these groups give off their protons and combine with formaldehyde. In buffered neutral formalin (with pH 7) amino groups are discharged and react avidly with formaldehyde. This difference underlines the importance of pH in penetration. For example, the amount of formaldehyde bound to collagen increase from 0.05 mmol/g at pH 4 to 0.4 mmol at pH 7 to 8. Maximum cross-linking, occurs in the moderate alkaline pH range and no increase in mild alkaline (pH 5.5).[7]
3. Volume of solution will ensure that the tissue penetration occurs from all surfaces ensuring the deeper penetration. As penetration is a function of concentration, by equation, 1, 2, 4, the concentration of the fluid at the advancing front is low, drawing much more influx of fluid in to the tissue. Moreover, contribution of osmotic driven forces, capillary driven forces will draw in more fluid in to the tissue causing higher penetration.[1,7]
4. With more time given, the depth of penetration will increase. As length is present in the denominator in equation 1, the increase in i, will be drawing more fluid in to the tissue. Medavar's formula given by the linear proportional increase of square root of time, with the coefficient of diffusion being constant.[15] However, it should be borne that the diffusion constant is subjected to variability too. Literature reports of too many values of such constants.[16]
5. With increase in pressure, the penetration of the solution is also rapidly increased.[17]
6. Presence of capillary vessels or muscle fibers has been observed to increase the rate of penetration. This

phenomenon is probably linked to the surface wetting or capillary action of the vessels and fibers.[18]

7. Increase in surface area of the tissue is expected to increase the rate of penetration. Presence of crypts, vessels and clefts appear to increase the rate of penetration.[18]
8. It is reported that the concentrated formaldehyde consistently penetrated more rapidly than less concentrated ones. The diffusion coefficient factor varied with concentration, but the logarithmic exponent remained unchanged. At the initial contact, the rate of penetration would be normal. As the tissue interacts with formaldehyde, concentration will drop to a hypothetical extent that when no further penetration occurred. In practical tissue fixation, hence it may be beneficial to use adequate quantities of fixing fluid or by re-immersion in fresh fluid.[1,18]

Factors influencing fixation

The single most important criterion that influences rate of fixation is the rate of shift of the equilibrium in favor of formaldehyde.[1] The factors that could influence the fixation include time, pH, temperature and viscosity.[18]

The absorption of formaldehyde is a function of its rapid utilization in the equilibrium equation. Subsequently, the kinetics of the dehydration of methylene glycol is an important rate limiting step in this reaction. In an experiment to determine the influence of pH on this reaction, it was observed that after a gradual increase with pH there is a sharp upturn of the pH corresponding to a decrease of the concentration of hydrogen ions. Indicating after a critical point in the range of pH of 7-8, it stabilizes. The dehydration rate probably is related to the amount of hydroxyl ion (H^+ or pH) in the tissue. When the tissue's alkalinity is utilized, the reaction would change and retard the fixation rate.[1]

In strong acidic environment, the primary amines (NH_2) combine with H^+ to form NH_3, carboxyl group (COO^-) loses their charge while OH^- group becomes less reactive. The presence of blood and proteins in tissue renders the free formaldehyde ineffective. In alkaline environment, the methylene glycol and other polymers break down to lesser degree causing the active formaldehyde.[1,17-19]

When carbonyl formaldehyde is formed, it rapidly establishes equilibrium with methylene glycol. The equilibrium constant for the methylene glycol to carbonyl formaldehyde interconversion is $4 \times 10^{-4}\ S^{-1}$. This indicates in this system, amount of carbonyl formaldehyde in any given aqueous formaldehyde solution will be low as most of them will be converted to methylene glycol.[1]

Owing to exhaustion of free formaldehyde, after the initial phase of primary reaction of fixation, it takes longer time for the secondary reaction to occur. Usually for smaller specimen, the longer they are stored more methylene bridges are formed and they are fixed permanently. Whereas for larger specimens such as those stored, the reaction continues for days together.

The reaction rate of the hydration of formaldehyde as obtained from measuring the chemically enhanced absorption of formaldehyde gas into water in a stirred cell with a plane gas liquid interface, and mathematically modeling of the transfer processes. At the conditions at temperatures of 293-333 K (22°C - 60°C) and at pH values between 5 and 7, the rate is found as $K_h = 2.04 \times 105 \times e\text{-}2936/T\ s\text{-}1$.[13] The formula[13] was given as:

$$\text{Chemical hydration constant for formaldehyde} = \frac{\text{Rate constant for reaction for formaldehyde}}{\text{Rate of dehydration of Methylene glycol}}$$

From this it can be safely assumed that rate of interconversion is a function of temperature. Increase in temperature has also shown to increase the fixation by formaldehyde. In a study using specific peptides coupled to slides, which were fixed by immersing the slides in 10% neutral buffered formalin for various lengths of time: 20 min or 1, 6, or 16 to 20 h (overnight). It was found out that the peptides, depending upon type, required between 6 and 16 h for complete fixation and loss of immunoreactivity. The fixative need not penetrate to any depth, since the peptides were on a molecular layer on the glass microscope slide. Consequently, the time was solely a reflection of the kinetics of a chemical reaction of formaldehyde with proteins at room temperature. Furthermore, the study identified that raising the formalin fixation temperature to 42°C significantly reduced the required incubation time. However, no reason for prolonged time taken for fixation has been given.[20]

Moreover, Increase in temperature will favor the disassociation of formaldehyde from the polymers. As the protein's action is related to temperature, the formaldehyde will be rapidly utilized to form bonds. This tilts the formaldehyde-methylene glycol in favor of formaldehyde, releasing more formaldehyde, hence the faster reaction.[1,8,17-21]

It has been shown that the addition of elevated pressure to conventional formaldehyde fixation improves the diffusion of formaldehyde throughout the tissue, as well as accelerates the fixation process.[17] It is conventionally advised to have an ideal volume of formaldehyde solution in ratio of 1: 25 with a minimum ratio of 1:10. This ensures that the amount of formaldehyde remains large to ensure the continuation of reaction in favor of conversion of formaldehyde. When low volumes of solutions are used, probably the minimally present formaldehyde molecules used and bank on the conversion of methylene glycol conversion for formaldehyde.[1,17,19-21]

It is assumed that the formaldehyde cross-linking is that the

fixed structures accurately reflect molecular relationships in the living cell. In a recent study, it is reported that such an assumption becomes invalid when intermolecular contacts are short-lived.[22]

CONCLUSION

Formaldehyde has an extensive and long history for fixation of tissues for diagnostic purposes. However, few aspects of the chemistry and physics of formaldehyde fixation have not been adequately explained. An attempt has been made to explain the physics and chemistry of formaldehyde fixation.

It has been reported that tissue, when stored indefinitely in formalin, does not yield good antigen and hence may give false negative results.[21] It is always useful to store the necessary tissue as paraffin blocks, though the validity of the method is still debated.[8] The chemistry and physics behind these phenomenon have been discussed. The discussion presented underlines the advantage of the routine use of buffered formalin instead of conventional formalin. This renders the penetration and fixation more effective that the conventional formalin. With the more extensive use of immunohistochemistry and other molecular techniques for diagnostic purposes, the modalities of formaldehyde fixation have to be understood before formaldehyde fixation is attempted. The action of formaldehyde in its slow formation of covalent bonds in aqueous solution yet rapid diffusion in tissue has to be effectively harnessed and employed for effective fixation, as well as antigen retrieval. The factors can be modified appropriately as discussed for effective fixation. The tissue processing shall not be done mechanically but after a careful thought process and warrants due attention to reduce errors, as well as to make the tissue processing faster.

ACKNOWLEDGEMENT

The authors would like to thank Dr. S. Ramachandran, Principal, Ragas Dental College and Prof. Dr. A. Kanagaraj, Chairman of Jaya Group of Institution, Chennai for their constant support and encouragement.

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How to cite this article: Thavarajah R, Mudimbaimannar VK, Elizabeth J, Rao UK, Ranganathan K. Chemical and physical basics of routine formaldehyde fixation. J Oral Maxillofac Pathol 2012;16:400-5.

Source of Support: Nil. **Conflict of Interest:** None declared.